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Characterization of turkey and chicken ghrelin genes, and regulation of ghrelin and ghrelin receptor mRNA levels in broiler chickens

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Abstract

Ghrelin, a peptide hormone produced by the stomach in mammals, stimulates growth hormone release and food intake. Recently, ghrelin was identified and characterized in chicken proventriculus and shown to stimulate growth hormone release but inhibit feed intake. The purpose of this work was to identify and further characterize the ghrelin gene in chickens and in turkeys. Using molecular cloning techniques we have sequenced cDNAs corresponding to chicken (White Leghorn) and turkey ghrelin mRNAs. A total of 844 (chicken) or 869 (turkey) bases including the complete coding regions (CDS), and the 5'- and 3'-untranslated regions (UTRs) were determined. Nucleotide sequence (CDS) predicted a 116 amino acid precursor protein (preproghrelin) for both the chicken and the turkey that demonstrated complete conservation of an N-terminal 'active core' (GSSF) including a serine (position 3 of the mature hormone) known to be a modification (acylation) site important for ghrelin bioactivity. Additional nucleotide sequence was found in the 5'-UTRs of both Leghorn and turkey cDNAs that was not present in broilers or the red jungle fowl. The turkey ghrelin gene, sequenced from genomic DNA templates, contained five exons and four introns, a structure similar to mammalian and chicken ghrelin genes. Ghrelin was highly expressed in proventriculus with much lower levels of expression in other tissues such as pancreas, brain, and intestine. RT-PCR was used to quantify ghrelin mRNA levels relative to 18S rRNA in 3-week-old male broiler chickens. The level of ghrelin mRNA increased in proventriculus in response to fasting but did not decline with subsequent refeeding. Plasma ghrelin levels did not change significantly in response to fasting or refeeding and did not appear to reflect changes in proventriculus ghrelin mRNA levels. Ghrelin mRNA levels declined in broiler pancreas after a 48 h fast and increased upon refeeding. Expression of the gene encoding the receptor for ghrelin (growth hormone secretagogue receptor, GHS-R) and a variant form was detected in a variety of tissues collected from 3-week-old male broiler chickens possibly suggesting autocrine/paracrine effects. These results offer new information about the avian ghrelin and ghrelin receptor genes and the potential role that this system might play in regulating feed intake and energy balance in poultry.

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Keywords: Chicken; Energy metabolism; Feed intake; Gene expression; Ghrelin; Growth hormone; Growth hormone secretagogue receptor; Pancreas; Proventriculus; Turkey

1. Introduction

The ghrelin system consists of a small acylated peptide hormone and its cognate receptor, the growth hormone secretagogue receptor (GHS-R). As a member of the G-protein-coupled receptor superfamily, GHS-R signals via

activation of the protein kinase C system and hydrolysis of phosphatidylinositol (phospholipase-IP3 pathway) resulting in the elevation of intracellular calcium levels (Korbonits et al., 2004; Ueno et al., 2005). Ghrelin was originally isolated from mammalian stomach tissue and subsequently shown to be a naturally occurring ligand for GHS-R (Kojima et al., 1999). This unique acylated peptide hormone has now been identified in several non-mammalian vertebrate species such as bullfrog

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(Kaiya et al., 2001), chicken (Kaiya et al., 2002), and fish (Kaiya et al., 2003; Parhar et al., 2003; Unniappan et al., 2002; Unniappan and Peter, 2005). Although ghrelin is predominantly a product of gastric tissue, expression has been detected in a variety of tissue sites including intestine, pancreas, placenta, brain, pituitary, hypothalamus, lung, cells of the immune system, kidney, and thyroid gland (Korbonits et al., 2004; Ueno et al., 2005). Similarly, GHS-R expression occurs in a variety of sites including the central nervous system and peripheral tissues indicating the potential for systemic (endocrine) as well as localized (autocrine/paracrine) functioning of this important hormone system (Korbonits et al., 2004; Ueno et al., 2005).

In mammals, ghrelin plays important roles in regulating growth hormone (GH) release from the pituitary gland as well as in regulating appetite, energy balance, adiposity, gastrointestinal function, immune function, reproductive function, and cardiovascular function (Korbonits et al., 2004; Tena-Sempere, 2005; Ueno et al., 2005; Van der Lely et al., 2004). Ghrelin and its receptor have been identified in the pancreas (Kageyama et al., 2005) where the ghrelin system has been suggested to function as part of a physiological control mechanism (calcium signaling via GHS-R) regulating β cell development and insulin secretion, as well as exocrine function in the pancreas (Date et al., 2002; Dezaki et al., 2004; Lai et al., 2005) and in the central regulation of pancreatic exocrine secretion mediated via vagal nerve efferent pathways (Sato et al., 2003). Moreover, a new ghrelin-secreting cell type (epsilon cell) has been described in pancreatic islet tissue (Prado et al., 2004). In general, as a regulatory component of the complex brain–gut anabolic neuroendocrine network controlling food intake, energy balance, and body weight, ghrelin may play a fundamental role in coordinating energy needs with the processes involved in growth.

Considerably less is known concerning the ghrelin system in avian species as compared to mammals. Chicken ghrelin has been purified from proventriculus and found to contain 26 amino acids (as opposed to 28 in mammals) with the third serine residue from the N-terminus of the mature peptide being acylated with *n*-octanoic or *n*-decanoic acid (Kaiya et al., 2002). Fatty acylation has been reported to confer bioactivity to the modified peptide hormone by enabling it to bind to, and signal through, the GHS-R (Kaiya et al., 2002). A gene homologue that codes for the chicken GHS-R (GHS-R1a) and a variant form (GHS-R1aV also designated GHS-R1c, truncated in the sixth transmembrane domain by alternative splicing of the gene transcript) have been cloned and their expression pattern in various tissues studied (Geelissen et al., 2003; Tanaka et al., 2003). Ghrelin immuno-positive cells have been detected in the hypothalamus, proventriculus, and gastrointestinal tract of birds (Ahmed and Harvey, 2002; Neglia et al., 2004, 2005; Wada et al., 2003; Yamato et al., 2005). There have been several reports that injections of acylated ghrelin peptide increase pituitary GH release (Ahmed and Harvey, 2002; Baudet and Harvey, 2003; Kaiya et al., 2002), increase plasma corticosterone levels (Kaiya et al.,

2002; Saito et al., 2005), and inhibit feeding behavior (Furuse et al., 2001; Geelissen et al., 2005; Kaiya et al., 2002; Saito et al., 2002; Saito et al., 2005) in chickens. Recently, conflicting (stimulatory or inhibitory) effects of peripheral versus central ghrelin administration were reported on food intake in the Japanese quail (Shousha et al., 2005). However, the exact role that the ghrelin system plays in birds still remains largely unknown. The purpose of this work was to further characterize the ghrelin gene in chickens and turkeys, and to follow its expression as well as the expression of GHS-R in 3-week-old broiler chickens under different energy states with the goal of better understanding the function of the ghrelin system in different avian species.

2. Materials and methods

2.1. Animals and materials

Chicks (*Gallus gallus*, broiler, and White Leghorn) and turkey (*Meleagris gallapavo*) poults were reared from hatching to 3 weeks of age in heated battery/brooder units. All birds received a standard starter poultry ration and water ad libitum. At 3 weeks, tissue samples were collected and snap-frozen in liquid nitrogen prior to RNA isolation. In a separate experiment, 30 broiler chickens (3-week-old males) were divided into five groups of six birds each and subjected to the following treatments: (1) fed ad libitum, (control), (2) fasted for 24 h (S24), (3) fasted for 48 h (S48), (4) fasted for 24 h and refed for 24 h (S24-RF24), and (5) fasted for 48 h and refed for 24 h (S48-RF24). All birds had free access to water throughout the experimental period. Upon termination of the experiment, samples of pancreas, whole brain, and proventriculus were collected and snap-frozen in liquid nitrogen prior to RNA isolation. Total RNA was isolated from tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA). Blood was collected by heart puncture. Following centrifugation (8000g 10 min at 4°C) plasma was collected and stored at –80°C prior to analysis. Genomic DNA was extracted from 50 μ l of whole blood using the Easy DNA extraction kit (Invitrogen). All protocols involving the use of animals received prior approval from the Beltsville Animal Care and Use Committee.

2.2. Nucleotide sequencing

Total RNA from proventriculus and primer-directed RT-PCR were used to generate turkey and chicken ghrelin cDNAs that were sequenced. Primer sets based on sequence reported for the broiler chicken (GenBank Accession No. AB075215) were initially used to generate a series of overlapping PCR products. The sequence of the turkey ghrelin gene was derived from genomic DNA templates using a primer-directed PCR strategy. PCR products were evaluated by agarose gel (1.5%) electrophoresis, and bands of the appropriate size were excised from the gel and purified using a GenElute gel extraction kit (Sigma Chemical, St. Louis, MO) or purified directly from the PCR sample by a

GenElute PCR clean-up kit (Sigma). Purified PCR products were subjected to bi-directional automated fluorescent DNA sequencing utilizing a Beckman Coulter CEQ 8000XL Genetic Analysis System using the dye terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA).

2.3. Rapid amplification of cDNA ends

Total RNA from proventriculus (1.0 µg) was used to prepare 3'- and 5'-rapid amplification of cDNA ends (RACE) ready cDNA using the SMART RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA). PCR was performed using Platinum *Taq* DNA polymerase with 3.5 mM Mg²⁺ (Invitrogen) and touchdown PCR. The following gene specific 3'- and 5'-RACE primers initially derived from the sequence of chicken and turkey ghrelin cDNA fragments were used:

3'-RACE (chicken): 5'-CCTTGGGACAGAACTGCTC-3'
 3'-RACE (turkey): 5'-AAGCCCCGCATATAAAAACA-3'
 5'-RACE (chicken): 5'-CACCAATTTCAAAGGAA CG-3'
 5'-RACE (turkey): 5'-CTCCAATGCTTGCCCATATT-3'

2.4. Cloning

Cloning of selected PCR products containing both the 5'- and 3'-untranslated regions (UTRs) was performed using a TOPO TA Cloning kit with a pCR 2.1 TOPO vector (Invitrogen). The inserted DNA was sequenced using M13 forward and reverse primers.

2.5. Ghrelin gene promoter analysis

Using nucleotide sequence for broiler (GenBank Accession No. AB075215) and Leghorn (GenBank Accession No. AY299454) ghrelin genes, BLAST-N searches of the chicken genome (http://www.ensembl.org/Gallus_gallus/index.html) were performed. The chromosomal location of the ghrelin gene was established as was the sequence of the 5'-flanking region (promoter). Promoter sequence was identified to include 2000 bp of genomic sequence upstream from the start codon (ATG) which was set at +1. This region was scanned for a TATA box and putative transcription factor binding sites with the aid of the TFSEARCH program (<http://www.cbrc.jp/research/db/TFSEARCH.html>).

2.6. RT-PCR

Reverse transcription (RT) reactions (20 µl) consisted of: 1.0 µg total RNA, 50 U Superscript II reverse transcriptase (Invitrogen), 40 U of an RNase inhibitor (Invitrogen), 0.5 mM dNTPs, and 100 ng random hexamer primers. Polymerase chain reaction (PCR) was performed in 25 µl reactions containing: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.0 U Platinum *Taq* DNA polymerase (Invitrogen), 0.2 mM dNTPs, 2.0 mM Mg²⁺, 10 pmol of each gene specific primer (Table 1), 5 pmol of an appropriate mixture of primers and competimers specific for 18S rRNA (QuantumRNA Universal 18S Standards kit, Ambion, Austin, TX) and 1 µl of the RT reaction. Thermal cycling parameters were 1 cycle 94 °C for 2 min, followed by 30 (ghrelin) or 35 (GHS-R) cycles, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min with a final extension at 72 °C for 8 min. Under these conditions, reactions were conducted within the linear phase of amplification for each PCR product. All PCR products were verified by direct sequencing to ensure fidelity of the amplification.

2.7. Capillary electrophoresis with laser induced fluorescence detection

Quantification of PCR products was accomplished using capillary electrophoresis with laser induced fluorescence detection (CE-LIF) as described previously (Richards and Poch, 2002). Aliquots (2 µl) of RT-PCR samples were first diluted 1:100 with deionized water. A P/ACE MDQ (Beckman Coulter) equipped with an argon ion LIF detector was used. Capillaries were 75 µm ID × 32 cm µSIL-DNA (Agilent Technologies, Palo Alto, CA). Enhance dye (Beckman Coulter) was added to the DNA separation buffer (Sigma, St. Louis, MO) to a final concentration of 0.5 µg/ml. Samples were loaded by electrokinetic injection at 3.5 kV for 5 s and run in reverse polarity at 8.1 kV for 5 min. P/ACE MDQ software (Beckman Coulter) was used to calculate peak areas for the PCR products separated by CE.

2.8. Quantitation of mRNA levels

The levels of ghrelin and GHS-R mRNA in different tissues were determined as the ratio of integrated peak area for each PCR product relative to that of the co-amplified

Table 1
PCR primers for analysis of chicken ghrelin and GHS-R mRNA levels by RT-PCR

Gene	Primer sequence	PCR product size (bp)
Ghrelin	Forward: 5'-CCTTGGGACAGAACTGCTC-3' Reverse: 5'-CACCAATTTCAAAGGAACG-3'	203
GHS-R (1a and 1aV) ^a	Forward: 5'-TTTTCCTGCCCCGATTCTG-3' Reverse: 5'-GCTTGGTGCTGGAGAGTCTT-3'	397 (1a) 349 (1aV)
GHS-R (total) ^b	Forward: 5'-AGCTGCACCTACTCCACCAT-3' Reverse: 5'-TTGTCCCTGATAATGGTGCTC-3'	395

^a Used to co-amplify GHS-R1a and GHS-R1aV as amplicons of different size.

^b Used to co-amplify GHS-R1a and GHS-R1aV as a single amplicon.

18S rRNA internal standard (Ambion). Values are presented as the mean \pm SEM of six individual determinations.

2.9. Hormone analyses

Plasma corticosterone was determined using a commercial kit purchased from MP Biomedicals, LLC (Irvine, CA). Plasma ghrelin was determined with a kit obtained from Linco Research, Inc. (St. Charles, MO). Insulin and growth hormone were measured as previously described (McMurtry et al., 1983; Vasilatos-Younken, 1986; respectively).

2.10. Statistical analysis

Gene expression and plasma hormone data were subjected to analysis of variance using the general linear models (GLM) procedure of SAS software (The SAS System for Windows, v. 8.2; SAS Institute, Cary, NC). Differences among individual means were evaluated using the Student–Newman–Keuls test option of the GLM procedure for SAS software. Significance for mean differences was set at $P < 0.05$.

3. Results

3.1. Structure of avian ghrelin genes

A portion of the ghrelin gene was isolated, sequenced, and characterized from turkey genomic DNA using a primer-directed polymerase chain reaction (PCR) molecular cloning strategy. Direct sequencing of overlapping PCR-generated DNA fragments identified and confirmed a total of 2615 contiguous bases corresponding to the portion of the gene that included the complete coding region (CDS), and the 5'- and 3'-untranslated regions (UTRs) of the corresponding mRNA transcript (GenBank Accession No. AY497549). The

Table 2

Species comparisons of ghrelin gene structure

Gene feature	Trout ^a	Chicken ^b	Turkey ^c	Mouse ^d	Human ^e
<i>Exon size (bp)</i>					
Exon 1	25	127 ^g	127	19	20 ⁱ
Exon 2 ^f	155 ^h	136	166	133	140
Exon 3 ^f	96	114	114	117	117
Exon 4 ^f	112	109	109	109	109
Exon 5 ^f	142	358	353	143	144
<i>Intron size (bp)</i>					
Intron 1	240	183	80	409	506 ⁱ
Intron 2	130	524	528	93	194
Intron 3	867	463	450	1942	2948
Intron 4	1503	692	688	783	809
<i>mRNA size (bp)</i>					
mRNA (cDNA)	535 ^h	844 ^g	869	521	530
Coding region	336 ^h	351	351	354	354
5'-UTR	57	155 ^g	185	44	52
3'-UTR	142	338	333	123	124

Sequence data were obtained as follows:

^a Rainbow trout (AB096919 and AB101443).

^b Chicken (AY299454 and AY303688).

^c Turkey (AY333783 and AY497549).

^d Mouse (NM_021488 and AB060078).

^e Human (AB029434 and AF296558).

^f These exons contain coding sequence.

^g Values are given for Leghorn chickens. Broiler chickens lack an 8 base sequence feature contained in exon 1 (5'-UTR) and thus are shorter.

^h Two mRNAs have been reported for the rainbow trout (Kaiya et al., 2003). One is 535 bp and another is 526 bp due to differential splicing of exon 2 within the CDS that results in the presence/absence of three amino acids (V¹³R¹⁴Q¹⁵) in the ghrelin protein.

ⁱ A small first exon has recently been identified for the human ghrelin gene (Nakai et al., 2004).

turkey ghrelin gene, like that in the chicken and various mammalian species, was found to consist of five exons (four of which contain coding region) and four introns (Fig. 1). Table 2 summarizes a comparison of different structural characteristics including exon and intron sizes as well as the

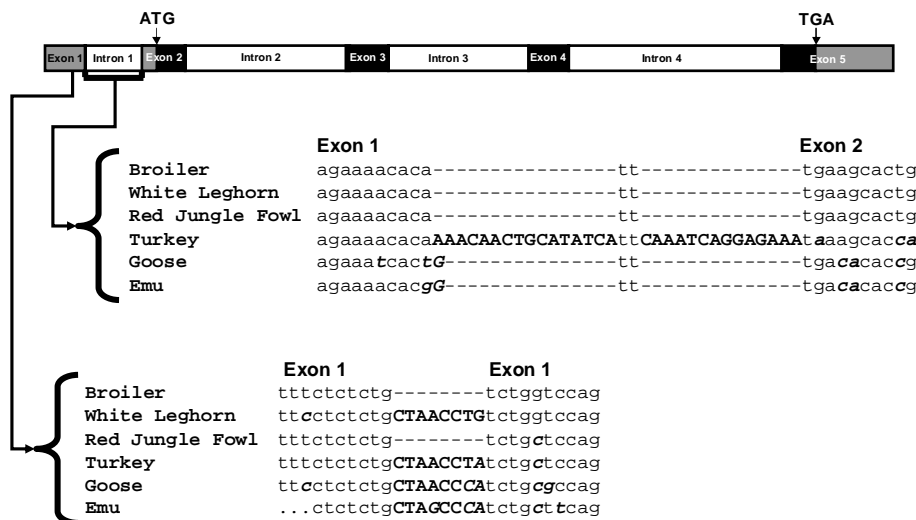


Fig. 1. General structure of the avian ghrelin gene based on sequence data obtained for the chicken (GenBank Accession No. AY303688) and turkey (GenBank Accession No. AY497549). The positioning of each of the five exons and four introns is indicated. The start codon (ATG, located in exon 2) and stop codon (TGA, located in exon 5) are also indicated with the black boxes designating coding region. The location of two different insertion/deletion (indel) features in the first two exons which contain 5'-untranslated region for different avian species is depicted.

size of different portions (CDS, 5', 3'-UTRs, etc.) of the mRNA transcripts from ghrelin genes of different species.

Fig. 2 depicts 5'-flanking sequence of the chicken ghrelin gene that contains the presumptive promoter region for this gene. A TATA box was detected 26 bp upstream from the start of exon 1 (transcription initiation site). In addition, evidence was found using a computer-assisted search (TFSEARCH) of 2000 bp upstream of the translation initiation site (ATG, set at +1) for putative transcription factor binding sites including: two cyclic AMP response element binding protein (CREB) sites, two AP-1 sites, one sterol regulatory binding protein-1 (SREBP-1) site, six C/EBP (CCAAT/enhancer binding protein) sites, and a number of SRY and SOX-5 sites among others.

3.2. Molecular cloning of chicken and turkey ghrelin cDNAs

Sequence of cDNAs derived from White Leghorn chicken (Fig. 3) and turkey (Fig. 4) ghrelin mRNAs was determined by molecular cloning involving primer-directed RT-PCR and 3'- and 5'-RACE. Both cDNAs contained an open reading frame of 354 bp encoding a 116 amino acid precursor protein, preproghrelin. A comparison of chicken versus turkey sequence demonstrated 91% homology at the nucleotide level and 93% homology at the amino acid level (data not shown). The presence or absence of an 8 bp insertion/deletion (indel) found in exon 1 (a non-coding exon containing 5'-UTR) distinguishes an egg laying strain (White Leghorn) from meat-type birds (broilers) and the ancestral strain (red jungle fowl). Turkeys also possess an 8 bp indel in the same position, as well as additional sequence at the junction of exons 1 and 2 (Fig. 1).

In both chickens and turkeys, the preproghrelin precursor was found to consist of a 23 amino acid signal peptide, the mature ghrelin peptide (26 amino acids), and a C-terminal extension peptide of 67 amino acids (Fig. 5). Comparisons of preproghrelin precursors from different avian species showed a high degree of amino acid sequence similarity with complete conservation of an 'active core' consisting of the first 4 N-terminal amino acids (GSSF) of the mature ghrelin peptide that contains the site (serine 3) of fatty acid acylation.

3.3. Expression of ghrelin and GHS-R genes

Ghrelin was expressed in a variety of tissues from 3-week-old broiler chickens, with the highest levels occurring in proventriculus, pancreas, brain, and small intestine (Fig. 6). Ghrelin receptor (GHS-R) was expressed in all of the tissues examined indicating the potential for widespread effects of ghrelin and the possibility of autocrine/paracrine effects of this hormone in chickens. Moreover, both isoforms (GHS-R1a and GHS-R1aV) were co-expressed; although there was some indication that GHS-R1a/GHS-R1aV ratio differed among tissues (Fig. 6).

Ghrelin mRNA level in proventriculus was significantly ($P > 0.05$) upregulated in 3-week-old broiler chickens in response to feed deprivation up to 48 h, but did not return to control levels with subsequent refeeding for 24 h (Fig. 7). In contrast, a 48 h fast significantly ($P < 0.05$) reduced ghrelin mRNA level in pancreas which was reversed by refeeding for 24 h. There was no significant ($P > 0.05$) effect of feed deprivation or refeeding on ghrelin mRNA levels in brain nor were there any significant effects on GHS-R mRNA levels in any of these tissues. However, the pancreas demonstrated an

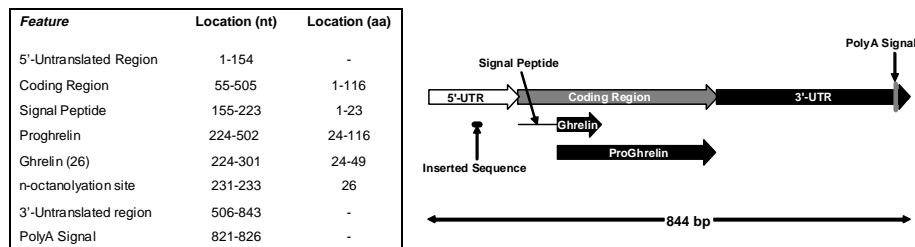
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-2000 GATACATGAA AATCTCCATG CATTCCCAAA GATGAAAAAG AGTAGCAGAA CGCATATGTA TTTTGCTTTA CAGTAATCTC AAACCTGTGT ATTACAAA
      <OCT-1 <C/EBP <C/EBP
-1900 CATCAACAAA TGAAATACA ATTGAAGAC TTTTITTTAG CCACAATTCT ATACTGTACC TCAGGTITTT GTTTAGACAA CAAAATAAAG TCTTATTGGT
      <SRY
-1800 TAAAGCCCTG TATGCTGTAT TTGTAATCT TTCACAGGGG TTTTATTATG AGGTTTCTTA TAGTCTGCCT TGACAGATGC CTTAGTGTGG CTCTCATGTA
      C/EBP
-1700 TAGTATTATG CTTTGTGAAG GCTAATAAAT GCCTATTCTG GCCAGAATCC ACAGTATACC ATGAAAATGC AAGGGGCTTC TGTTAGGACC CAGAAGCTCT
      <CDXA
-1600 CCAGTGGTTC ATCACTGCCT ACTTCTCTTG TGCTCCTCAG TGTTCTCTGC CAGCAGGCTG TCAGACAAAA GTCAAGACGG AGATCTAACT CTATAAGAAG
      <SRY
-1500 TTCTTAACCT AACTTTGTGG CAGAAGGGAT TTGATCCATT GCCTCTGATT GTTCTCAGCT GCCTAGTGGC AGTTAACCAGC TTTCTTGGTG CTACCCAGTC
      <SOX-5
-1400 AGCTGATACAT ATGGACATGT TTCAGAAATT TTCTTTTAAA CAATTGGATC ATTACACAAA TCTGATTTGA AACACAGCTG CAGTGTTTGA AAAAATCAGC
      <OCT-1 <SRY <C/EBP
-1300 TGGGAAGCAG TAATGTGAGA TGTGGAATCT GTGAAAGAGA ACAACATGGA ACTGCATCCC CAGAAAAAAA CTAGTTTCAA AACCTGCGCA TGTGCTAAAG
      <CDXA <MZF1 <GATA-1
-1200 AGACTGCTT CTTTGGCCAG AGCACAGTTA CTATGGTCAC TCTATGTAGC GATGCTCCAA GTTACATTTT CTCCAACAGA AAATCTGACT CAATAAATT
      <CDXA <AP-1
-1100 CCTTCTCAIT TCCGATGCTT TCCTCAATCC TACAATGAA GGTTTGTGAT CTGTAGCAAT GAATAAATCC CACAGATCAG CCACATCATT CAAGCTTAGC
      <BRN-2 <GATA-1
-1000 TGGAACACAA GCAACAGAAG GTAAATTTGT CATCCTTTAC TGTGTGTTCC TACTTCATTC CTATTATTCC TCAGGTTTGA AGACAATCCT CACAGGATGT
      <CDXA
-900 TTTCTAGAGC CTCAGGCATT GCTACAAGTA TTCATGACA TCTTCAAAAT GCAGTATTTT ATTTCCTAAAG TAAGAGAGAT GATGTTTCTA GGTAAATCTT
      <OCT-1
-800 TTGTTTTCGG TCTTTCAGCA AAGGACAACA AAATCTGATA GAACAGAACC CAGGAGAGAA TGTAGATGAG AGGAAGCACA CTTAGGGGCA AACACAGAA
      <SRY
-700 ACATGGTGTG TACTGCGAGC CAGGAATGGA GTTCTGCTTC TTTTCTGTTA CACCACGCCA AATAATGGTA AAAGGGCTCA GGACTTTCCA AAACATAGCT
      <C/EBP
-600 ATTACAGCTA CTAGTTTTTA GAAGGAATGA TGTATGCCA CAGCAGAAAA ACACAAAGAA CACACAATTG CCCACAAAT TTCATAATAC GTGAAAGGA
      CREB SRY <C/EBP
-500 ACTCAGCAGC AGATGTATGC AGTTTTCATG TTTTGTCAA CTGTTATCTT ACAAGCACAC CAGCTTTCCA AGGAATCTAA ATACACGTCA GACTGCATTT
      <GATA-1 <CREB
-400 CCAGAAATAT CACCGCACAG AACAAATATC CCATCCCTTA TATAAGGTGC TTCAGAAAGC AGCAGTTTGG TGAAGCAACA AAGGTAACCT GGTGAAGTTT
      SREBP-1 TATA Box Exon 1
-300 CAGAGCATTT CTAAGCTTTT GCCAGTTTTC CTCTGTAATT TCTCTCTGTC TGGTCCAGTC ATTACATCTC AGTTATAGAA GAAACACAG TAAGTAATCT
      Exon 2
-200 CTTTTCAGAC GATATATTGC CTTTCTGCAT GCAACAATTG TGCTTTCTTT CTATATTGTT TTGGAGAAAC AATTGCACAT CATTCTAATC AGGAGAACTA
-100 TTTTCACTGG CTTGGGATTC AACCAATATC ACAGATTAGA AAATATGTTT AATTTTAAAC TTGTGTTTTC AGTTTGAAGC ACTGCTCTAA GAAGACAACC
      +1 ATG

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Fig. 2. The nucleotide sequence of the 5'-flanking region of the chicken ghrelin gene. The translation start site (ATG) was set at +1. A TATA box motif was identified at -361 to -355. Putative binding sites for different transcription factors were identified and the sequence is highlighted by underlining. The sequence enclosed in boxes designates that for exons 1 and 2.

Chicken (White Leghorn) Preproghrelin mRNA



cDNA Nucleotide and Translated Amino Acid Sequences

1	TGAAGCAACA	AAGGTAACCT	GGTGAAGTTT	CAGAGCATTT	CTAAGCTTTT	GCCAGTTTTC
61	CTCTGTAATT	CCTCTCTGCT	AACCTG TCTG	GTCCAGTCAT	TACATCTCAG	TTATAGAAGA
(1)		Inserted sequence			M F L R V I L L G	
121	AAACACATTT	GAAGCACTGC	CTAACGAAGA	CAACCATGTT	TCTCAGAGTT	ATTCTGCTAG
(10)	I L L L S I L G T E T A L A				G S S F I	
181	GAATTCTCCT	TCTCAGCATC	CTTGGGACAG	AAACTGCTCT	GGCTGGCTCT	AGTTTITTA
(29)	S P T Y K N I Q Q Q K D T R K P T A R I					
241	GCCCCACATA	TAAAAACATA	CAGCAACAAA	AGGATACAAG	AAAACCAACA	GCAAGATTAC
(49)	H R R G T E S F W D T D E T E G E D D N					
301	ATCGCCGAGG	CACAGAAAGC	TTTGGGATA	CAGATGAAAC	AGAGGGAGAA	GATGACAATA
(69)	N S V D I K F N V P F E I G V K I T E R					
361	ACAGTGTGTA	TATCAAGTTT	AACGTTCTT	TTGAAATTGG	TGTCAAGATA	ACAGAAAGAG
(89)	E Y Q E Y G Q A L E K M L Q D I L A E N					
421	AGTATCAAGA	GTATGGACAA	GCACTGGAGA	AGATGCTACA	GGACATTCTT	GCAGAGAATG
(109)	A E E T R T K S *					
481	CTGAAGAAAC	TCGGACAAAA	AGCTGAAGCA	AGCTGAAGGA	CAACCACAGC	ACTAGATGGC
541	CTGATATAAG	TTTTACTTTA	AAACAAACT	ATGACTGAAT	TTAGATCTCT	AAGAAGAAAG
601	AAGCACATGA	ACTTGAGTAG	TTTGCTCTAA	GAAAAAGACA	AGAATCTACA	TGCAGAACTT
661	CAAAGGGCAA	TGTGAATCAC	AGGCTGACGT	GTAGGCTTAT	TTTATTTCCT	TCCTTAGTAT
721	CTACTGTGAT	GATTTTAAAT	GATATTTTGG	AAAGCTGTAT	GAATCCTTTC	TGAAACTGGT
781	GGAAACTTCA	TCTTACACTT	CTACAGACAT	CTGTAACAGA	GAAATAA AGCT	TATGCTGAAA
841	CTGC				PolyA Signal	

Fig. 3. Graphic representation and nucleotide and amino acid (AA) sequence for chicken (White Leghorn) preproghrelin cDNA (GenBank Accession No. AY299454). The locations and size (in bp or AA) of the untranslated regions (3'- and 5'-UTRs), coding region (CDS), signal peptide, the prohormone, and mature ghrelin peptide hormone are indicated. The nucleotide sequence of the CDS is highlighted by underlining. Translated AA sequence of the mature ghrelin hormone is highlighted by the shaded box. Numbering to the left identifies nucleotide and translated AA (bold font) numbers. Inserted nucleotide sequence and the polyA signal are denoted by bold-italic letters. The modification site (S26) for the fatty acylation of ghrelin is also indicated by underlined bold-italic font.

expression pattern similar to ghrelin with a decline in GHS-R mRNA level after a 48 h fast and a return to control levels upon refeeding.

3.4. Plasma hormone levels

An analysis of the data indicated that circulating ghrelin levels were not significantly ($P > 0.05$) altered by fasting and refeeding (Fig. 8). However, there was a trend toward increased plasma ghrelin concentration following 48 h of feed deprivation. Subsequent refeeding for 24 h decreased plasma ghrelin following a 48 h fast. Plasma GH levels were not significantly changed by fasting and refeeding and they did not show any apparent relationship with plasma levels of ghrelin (Fig. 8). Fasting significantly ($P < 0.05$) increased circulating levels of corticosterone, while subsequent refeeding returned the levels back to those comparable to the fed control birds (Fig. 8). Although the significant ($P < 0.05$) increase in plasma corticosterone level by 48 h of fasting was similar to the trend observed for circulating ghrelin, there was no similarity in the response of these two hormones to subsequent refeeding. In contrast, plasma insulin levels declined significantly

($P < 0.05$) in response to feed deprivation and returned to control levels upon refeeding (Fig. 8).

4. Discussion

4.1. Structure of avian ghrelin genes

This is the first report to characterize the sequence and structure of a turkey ghrelin gene (GenBank Accession No. AY497549). The turkey ghrelin gene containing the coding region and the 5'- and 3'-untranslated regions of the corresponding mRNA transcript was found to consist of five exons, four of which contain the entire coding region, and four introns. This structure is similar to ghrelin genes reported for the chicken, rainbow trout, and rodents (Kaiya et al., 2003; Nie et al., 2004; Tanaka et al., 2001). It has been reported that human and certain fish ghrelin genes consist of four exons and three introns (Hosoda et al., 2003; Parhar et al., 2003; Unniappan et al., 2002; Unniappan and Peter, 2005). These genes apparently lack the first exon which is short (only 19 bp) and non-coding (contains only 5'-UTR) in mouse and rat ghrelin genes (Tanaka et al., 2001).

Turkey Preproghrelin mRNA

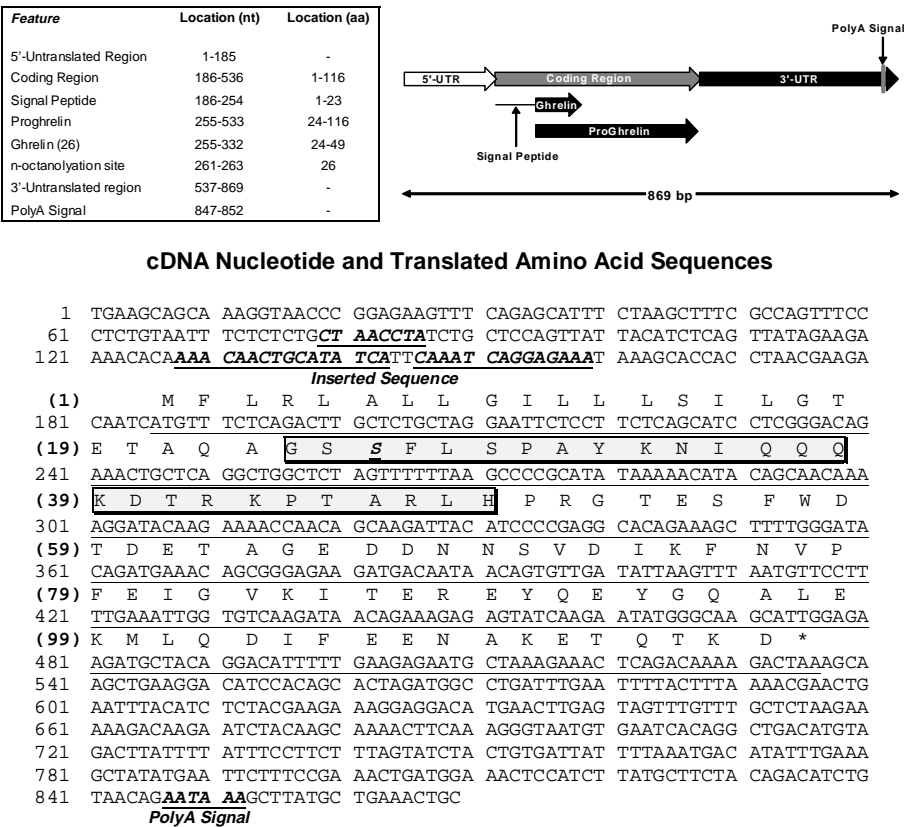


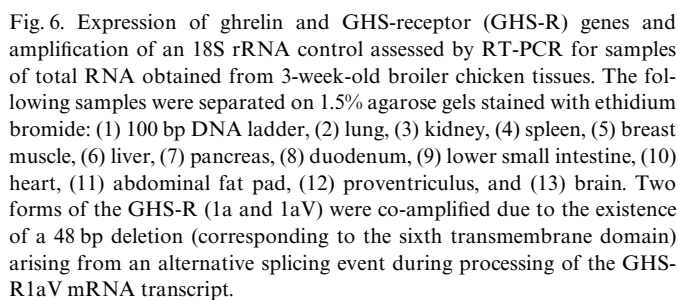
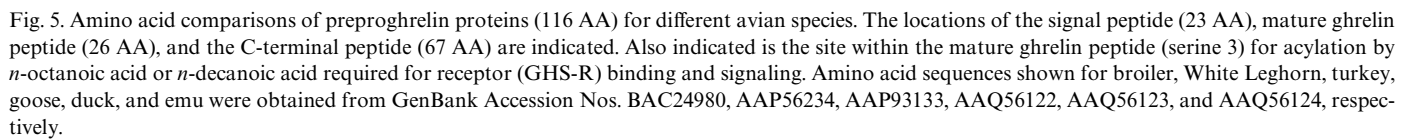
Fig. 4. Graphic representation and nucleotide and amino acid sequence for turkey preproghrelin cDNA (GenBank Accession Nos. AY333783). The locations and size (in bp or AA) of the untranslated regions (3'- and 5'-UTRs), coding region (CDS), signal peptide, the prohormone, and mature ghrelin peptide hormone are indicated. The nucleotide sequence of the CDS is highlighted by underlining. Translated AA sequence of the mature ghrelin hormone is highlighted by the shaded box. Numbering to the left identifies nucleotide and translated AA (bold font) numbers. Inserted nucleotide sequence and the polyA signal are denoted by bold-italic letters. The modification site (S26) for the fatty acylation of ghrelin is also indicated by underlined bold-italic font.

However, a recent study found evidence for a 20 bp putative non-coding first exon in the human ghrelin gene similar to the mouse (Nakai et al., 2004). Both chicken and turkey ghrelin genes have a larger first exon that contains exclusively 5'-UTR. The rainbow trout ghrelin gene, on the other hand, has a short (25 bp) first exon similar to rodents (Kaiya et al., 2003). What impact the size and/or presence of this first exon might have on ghrelin gene regulation remains to be shown.

During the course of sequencing turkey and chicken ghrelin genes, we observed a number of unique sequence variations. One particularly interesting feature was an 8 bp indel (insertion/deletion) found in the first exon of egg-type (White Leghorn) chickens that was absent in meat-type (broiler) chickens. This may be an important finding because these two strains differ markedly in their appetite and growth characteristics. The indel was also absent in red jungle fowl, an ancestral line for modern commercial chicken breeds. This unique indel feature has been reported to occur at low frequency in a study summarizing sequence polymorphisms in four chicken breeds (Nie et al., 2004, 2005). Moreover, sequence similar (but not identical) to the chicken indel and of the same size (8bp) was observed in the same location

(exon 1) in the turkey, goose, and emu (Fig. 1). The turkey ghrelin gene exhibited additional sequence (30 bp) at the junction of exons 1 and 2 that was not observed in any other avian species for which ghrelin sequence has been reported. Both of these sequence variations occur outside of the coding region of the mRNA transcript and their effect on ghrelin gene function remains to be determined. However, Nie et al. (2004) reported a single nucleotide polymorphism (SNP, C223G) occurring in the 5'-UTR of the chicken ghrelin gene that determined the presence or absence of a specific transcription factor (serum response factor) binding site that they suggested might influence expression of the ghrelin gene. They also found that the majority of the SNPs occurring in the chicken ghrelin gene were located in the four introns. Moreover, there were no significant differences in these sequence variations between egg layers and meat-type chickens (Nie et al., 2004).

We (GenBank Accession No. AY299454) and others (Nie et al., 2004, 2005) have reported a SNP (A/G) found in exon 5 (within the coding region) of the chicken ghrelin gene that results in an amino acid change (Gln113Arg). However, this change would affect the C-terminal peptide region of the prohormone and not the mature ghrelin



Analysis of the draft chicken genome sequence indicated that the ghrelin gene is located on chromosome 12. Flanking (5') sequence was examined in an attempt to determine characteristics of the putative promoter region and perhaps shed some light on factors that might regulate ghrelin gene expression in chickens. Little is known about transcriptional regulation of the ghrelin gene in mammals (Kishimoto et al., 2003; Nakai et al., 2004). Nothing is currently known about ghrelin gene regulation in birds. Glucagon and its second messenger, cAMP, have been reported to enhance human ghrelin gene transcription and this has been suggested as a possible mechanism that leads to increased plasma ghrelin levels in response to fasting (Kishimoto et al., 2003). Similarly, growth hormone releasing hormone (GHRH) was found to upregulate ghrelin gene transcription in the pituitary via a cAMP-signaling cascade (Kamegai et al., 2001). Our finding of two cAMP response element binding protein (CREB) sites in the putative promoter region of the chicken ghrelin gene would be consistent with the proposed gene regulation mechanism involving cAMP. Although an SREBP-1 site located just upstream of the TATA box is intriguing, a role for this transcription factor in ghrelin gene regulation has not been reported previously in any species. The presence of multiple SRY and SOX-5 sites could indicate their potential role in regulation of ghrelin gene expression specifically in the testis (Tena-Sempere, 2005); however, to date, no SRY gene homologue has been found in birds (Mizuno et al., 2002). In

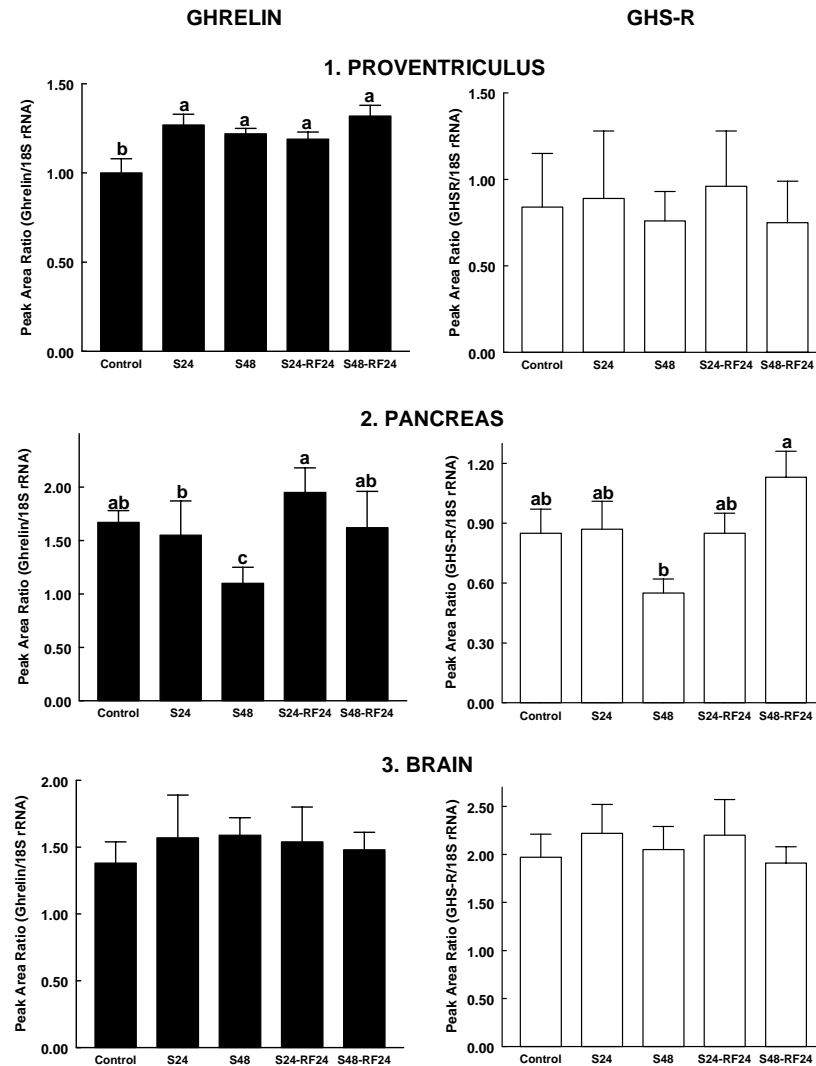


Fig. 7. Ghrelin and GHS-R mRNA levels in proventriculus, pancreas and brain tissue from 3-week-old broiler chickens subjected to periods of feed deprivation of 24 or 48 h (S24, S48) or refed for 24 h after a prior period of feed deprivation (S24-RF24, S48-RF24). Control chickens were fed ad libitum throughout. RT-PCR was used to quantify the mRNA level relative to an 18S rRNA internal standard. Values represent means \pm SEM of six determinations. Different letters above each bar denote statistically significant ($P < 0.05$) differences for mean comparisons.

general, the mechanisms and factors that regulate ghrelin gene transcription in the chicken remain to be clarified. Because of the high nucleotide sequence homology with the chicken ghrelin gene and the availability of draft genome sequence for the chicken, it is likely that a comparative genomics approach can be effectively utilized to clone additional regions of the turkey ghrelin gene such as the promoter region containing putative transcription factor binding sites. Such information would be useful in determining factors that affect turkey ghrelin gene transcription.

4.2. Molecular cloning of chicken and turkey ghrelin cDNAs

Full-length cDNAs corresponding to chicken (White Leghorn) and turkey ghrelin mRNAs have been cloned and sequenced (GenBank Accession Nos. AY299454 and AY333783). In the chicken, the mature ghrelin peptide consists of 26 amino acids and is processed from the pre-

cursor at the dibasic sequence Arg-Arg (RR) located at the C-terminal end of the peptide (Kaiya et al., 2002). In the turkey this sequence is Pro-Arg (PR), the same as human ghrelin, and the change may indicate a potential difference in the size of the mature turkey ghrelin peptide (28 vs. 26 amino acids) as compared to the chicken due to different C-terminal end proteolytic processing. All other avian species reported to date contain the Arg-Arg (RR) sequence at the C-terminal end of the ghrelin peptide which makes the turkey unique among avian species in this aspect. At the N-terminal end of the mature ghrelin peptide there is complete conservation of the first seven amino acids including a serine residue at position 3 (site of *n*-octanoylation) for all avian species. One amino acid change was observed in chickens (White Leghorn) due to a SNP. That was the Gln113Arg (Q/R) change in the C-terminal peptide of the precursor protein (Nie et al., 2004, 2005).

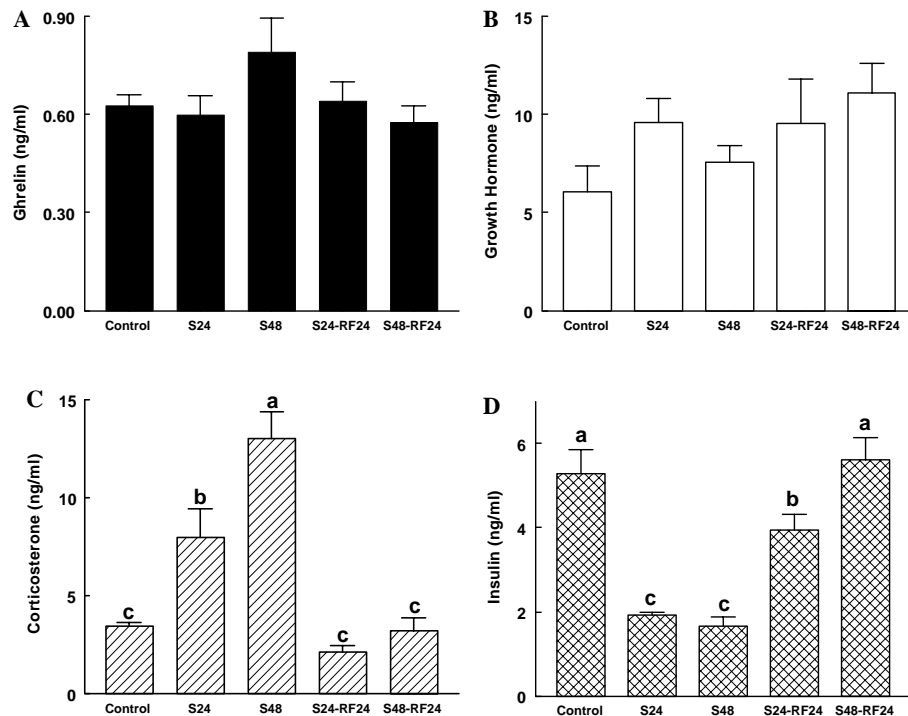


Fig. 8. Plasma ghrelin (A), growth hormone (B), corticosterone (C) and insulin (D) levels determined for 3-week-old broiler chickens subjected to periods of feed deprivation of 24 or 48 h (S24, S48) or refed for 24 h after a prior period of feed deprivation (S24RF24, S48RF24). Control chickens received feed ad libitum throughout. Values represent means \pm SEM of six determinations. Different letters above each bar denote statistically significant ($P < 0.05$) differences for mean comparisons.

4.3. Expression of ghrelin and GHS-R genes

Ghrelin was expressed in all tissues examined from 3-week-old broiler chickens. Proventriculus showed the highest expression followed by pancreas, brain, and intestine. This tissue expression pattern was more widespread than previously reported for chickens, but is consistent with what has been reported in other species (Korbonits et al., 2004; Ueno et al., 2005; Unniappan and Peter, 2005). Kaiya et al. (2002) reported ghrelin expression in proventriculus, brain, lung, spleen and intestine in 8-day-old chickens using RT-PCR. Saito et al. (2005) used RT-PCR to quantify ghrelin mRNA levels in different regions of the brain and found that the corpus striatum expressed the highest levels followed by the cerebellum, the optic lobes, and the brainstem. Wada et al. (2003) observed ghrelin mRNA expression only in the proventriculus of newly hatched Leghorn chicks, whereas in adult chickens, mRNA expression was also detected in duodenum. Interestingly, they observed that mRNA and protein expression were similar in cells from adult chickens, whereas in newly hatched chicks there was higher mRNA as compared to protein expression.

Expression of two GHS-R mRNAs (GHS-R1a and GHS-R1aV) was detected in all tissues examined from 3-week-old broiler chickens in this study. The highest levels of expression of GHS-R1a and GHS-R1aV were in brain, spleen, intestine, proventriculus, and liver. This is similar to the tissue expression patterns reported previously for 8-week-old White Leghorn chickens (Tanaka et al., 2003) and

10-day-old broiler chicks (Geelissen et al., 2003). In addition, there was evidence for differential ratios of GHS-R1a/GHS-R1aV expression among the tissues studied, although those tissues that expressed higher levels of GHS-R1a also showed higher expression of the variant form of the receptor and in the majority of tissues GHS-R1a was preferentially expressed. These findings may reflect differential tissue sensitivity to ghrelin since the GHS-R1aV variant which, in chickens, lacks a portion of the sixth transmembrane domain due to a 48 bp deletion, has been reported to be non-functional and thus incapable of signal transduction (Geelissen et al., 2003; Tanaka et al., 2003). However, the actual physiological function, if any, of the variant form of the receptor has yet to be determined. Since ghrelin is expressed in many of the same tissues where GHS-R1a is expressed, autocrine or paracrine in addition to the endocrine effects of ghrelin on these tissues have been suggested (Tanaka et al., 2003).

We also determined changes in ghrelin and GHS-R mRNA levels in proventriculus, pancreas and brain under different energy balance states created by fasting and refeeding of 3-week-old broiler chickens. Fasting (negative energy balance) did elevate ghrelin gene expression in proventriculus; however, refeeding (positive energy balance) did not reduce expression levels. This expression pattern clearly differed from that observed in rodents in which fasting significantly upregulated and subsequent refeeding significantly downregulated ghrelin expression in the stomach (Toshinai et al., 2001). There were no significant effects of

fasting or refeeding on GHS-R mRNA levels in the proventriculus, pancreas or brain in our study. It has been proposed that ghrelin is upregulated under conditions of negative energy balance to act as an anabolic signal in rats that promotes the acquisition (increased feed intake) and storage of energy to counteract the negative energy status (Toshinai et al., 2001).

We did observe a significant reduction in pancreatic ghrelin mRNA level following 48 h of fasting and subsequent refeeding led to an increase back to control levels. There was also a similar trend, although not significant, in GHS-R mRNA levels in the pancreas. Localized ghrelin expression in pancreatic epsilon (i.e., ghrelin) cells and the expression of GHS-R in β cells have been linked to regulating insulin secretion from islet tissue (Date et al., 2002; Kageyama et al., 2005). At present, it is unclear what role, if any, ghrelin and its receptor might play in regulating pancreatic insulin secretion in chickens.

In the brain, there were no significant effects on ghrelin or GHS-R mRNA levels in response to altered energy balance. Taken together, our findings concerning the broiler chicken suggest that the ghrelin system (i.e., ghrelin and GHS-R) is regulated differently than in mammals and thus may reflect differences in the effects of ghrelin on food intake in birds as compared to mammals.

4.4. Plasma hormone levels

Plasma ghrelin levels did not change markedly during fasting and refeeding with the exception of broilers fasted for 48 h. In mammals, the stomach has been reported to be the major site of ghrelin biosynthesis and secretion and plasma levels are determined by feeding state (Ariyasu et al., 2001). It is not known which tissue(s) in birds contributes to maintaining plasma ghrelin levels. However, the high level of ghrelin expression in proventriculus is consistent with a potential role in regulating circulating levels of ghrelin in birds (Kaiya et al., 2002). While it is generally recognized that plasma ghrelin levels in mammals are increased by fasting and reduced by refeeding, the regulatory mechanisms governing these processes have not yet been clarified (Ariyasu et al., 2001; Toshinai et al., 2001; Tschöp et al., 2000). Studies with rodents have suggested that circulating ghrelin reflects acute feeding states and thus may serve as an indicator of short-term energy balance (Ariyasu et al., 2001). Yamato et al. (2005) reported a significant increase in plasma active (*n*-octanoylated) ghrelin levels by day 3 post-hatch commensurate with the onset of feeding in neonatal chicks and that fatty acid absorbed from the diet is directly used in the production of acylated ghrelin. Circulating levels of ghrelin detected in the 3-week-old broiler chickens used in this study did not appear to reflect changes in feeding state. However, Shousha et al. (2005) reported that fasting (24 h) increased plasma ghrelin fivefold whereas it was decreased by refeeding (3 h) following a 24 h fast in adult male Japanese quail. Moreover, both fasted and refed groups exhibited elevated plasma ghrelin

levels compared to free feeding controls. These results suggested that peripheral ghrelin promotes food intake rather than acting as a satiety signal in quail. This concept was supported by the fact that small exogenous doses of ghrelin administered ip actually promoted food intake in quail. In contrast, central (icv) administration of ghrelin always inhibited food intake (Shousha et al., 2005).

Ghrelin administration has been shown to transiently increase plasma GH concentration in young chickens (Ahmed and Harvey, 2002; Kaiya et al., 2002). Dispersed pituitary cells also responded to ghrelin administration in vitro with a dose-related GH release (Baudet and Harvey, 2003). These results indicate a direct effect of ghrelin on pituitary somatotrophs possibly augmented by its effects on hypothalamic sites, both mediated by binding to and signaling through GHS-Rs (Baudet and Harvey, 2003; Kuhn et al., 2005). Thus, it has been demonstrated that ghrelin is a potent GH secretagogue in the chicken (Ahmed and Harvey, 2002; Baudet and Harvey, 2003; Kaiya et al., 2002). However, there have been no studies linking changes in endogenous plasma ghrelin with GH concentrations in birds. Therefore, the relationship of plasma ghrelin and GH levels over a longer timeframe has not been studied. In this study, no correlations were observed between circulating ghrelin and GH in response to dramatic changes in feeding state of 3-week-old broiler chickens. However, our single time point determinations (at 24 h intervals) may not have been sufficient to capture all of the acute fluctuations in plasma hormone levels, especially those that occurred right around the time of changes to the feeding state. Moreover, a multifactorial mechanism has been reported in the chicken that governs GH release into circulation involving the combined effects of the somatotropic-, thyrotropic-, and corticotropic-axes (Kuhn et al., 2005). Thus, the fact that ghrelin and GH levels were not correlated in this study does not preclude an important role for endogenous ghrelin in regulating sustained GH release in response to changes in feeding state. Such a relationship has been suggested as a potential mechanism for linking GH release to metabolic state (Kuhn et al., 2005). A recent report found that endogenous ghrelin declined during 4 days of fasting while GH levels increased in healthy women suggesting a reciprocal relationship between these two hormones (Koutkia et al., 2005). GH may participate in a negative feedback on ghrelin during short-term caloric restrictions. Together these results suggested an important physiological relationship between endogenous ghrelin and GH in response to changes in energy status in humans (Koutkia et al., 2005).

Kaiya et al. (2002) were the first to report a significant elevation of plasma corticosterone following the administration of a single iv dose of ghrelin to 8-day-old chickens. This effect was transitory and not sustained for more than 30 min. Saito et al. (2005) confirmed the ability of ghrelin (administered icv) to increase plasma corticosterone in 4- to 6-day-old chickens and went on to show that this effect was mediated through corticotropin releasing factor (CRF)-expressing neurons in the hypothalamus which bring about

an inhibition in food intake and activate the hypothalamic–pituitary–adrenal (HPA) axis leading to an increase in plasma corticosterone levels. Furthermore, they suggested that the release of corticosterone in response to ghrelin might play a role in regulation of energy homeostasis in birds. In our study, the dramatic changes in plasma corticosterone brought about by fasting and refeeding did not coincide with commensurate changes in plasma ghrelin. Feed restriction does increase neuronal NPY gene expression in the hypothalamus of broiler chickens although the mechanism for this response is not known (Boswell et al., 1999). The possibility of elevated plasma ghrelin in response to fasting as has been reported in quail (Shousha et al., 2005) and the activation of hypothalamic CRF-expressing neurons by ghrelin (Saito et al., 2005) could present a potential conflict. Two opposing hypothalamic circuits would be activated simultaneously, one orexigenic (NPY) the other anorexigenic (CRF). Shousha et al. (2005) have reported opposing effects of peripheral versus central ghrelin in Japanese quail on food intake. This apparent discrepancy may account for the lack of any significant sustained change in plasma ghrelin in response to fasting and refeeding observed in this study. Moreover, it would argue for a different mode of action for ghrelin in birds as compared to mammals where elevated plasma ghrelin in response to fasting is thought to activate NPY/AGRP-expressing neurons in the hypothalamus that stimulate food intake (Korbonits et al., 2004; Ueno et al., 2005; Van der Lely et al., 2004). Thus, the effects reported for the exogenous administration (iv or icv) of ghrelin on plasma corticosterone in the chicken are acute in nature and may not reflect sustained actions of endogenous circulating ghrelin. In support of this concept, Koutkia et al. (2005) found no relationship between changes in endogenous ghrelin and cortisol in healthy women during fasting.

Recent reports indicated an autocrine/paracrine role for ghrelin in regulating insulin secretion from the pancreas in rodents (Date et al., 2002; Kageyama et al., 2005). In our study, we showed that ghrelin and GHS-R genes were expressed in 3-week-old broiler pancreas and the level of ghrelin mRNA did appear to respond to changes in energy status during fasting and refeeding. Moreover, an apparent relationship between ghrelin mRNA levels in the pancreas and plasma insulin concentration was observed suggesting a possible positive influence of locally produced ghrelin on insulin secretion from the pancreas. This occurred despite the fact that changes in circulating ghrelin and insulin levels were not correlated. However, it remains to be demonstrated if in fact localized ghrelin production does play a role in regulating pancreatic insulin secretion in chickens.

In conclusion, our data provide additional information about the gene structure and regulation of ghrelin and its receptor in chickens and turkeys. This information provides additional insight into the potential role of the ghrelin system in regulating appetite and energy balance in poultry.

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